Pan-Viral Screening of Respiratory Tract Infections in Adults With and Without Asthma Reveals Unexpected Human Coronavirus and Human Rhinovirus Diversity

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(See the editorial commentary by Gern and Busse, on pages XXX–XX.)

   Background.   Between 50% and 80% of asthma exacerbations are associated with viral respiratory tract infections (RTIs), yet the influence of viral pathogen diversity on asthma outcomes is poorly understood because of the limited scope and throughput of conventional viral detection methods.

   Methods.   We investigated the capability of the Virochip, a DNA microarray–based viral detection platform, to characterize viral diversity in RTIs in adults with and without asthma.

   Results.   The Virochip detected viruses in a higher proportion of samples (65%) than did culture isolation (17%) while exhibiting high concordance (98%) with and comparable sensitivity (97%) and specificity (98%) to pathogen-specific polymerase chain reaction. A similar spectrum of viruses was identified in the RTIs of each patient subgroup; however, unexpected diversity among human coronaviruses (HCoVs) and human rhinoviruses (HRVs) was revealed. All but one of the HCoVs corresponded to the newly recognized HCoV-NL63 and HCoV-HKU1 viruses, and >20 different serotypes of HRVs were detected, including a set of 5 divergent isolates that formed a distinct genetic subgroup.

   Conclusions.   The Virochip can detect both known and novel variants of viral pathogens present in RTIs. Given the diversity detected here, larger-scale studies will be necessary to determine whether particular substrains of viruses confer an elevated risk of asthma exacerbation.

Between 50% and 80% of asthma exacerbations are associated with viral respiratory tract infections (RTIs) [1, 2]. In young children, a variety of viral pathogens accompany wheezing episodes, most notably respira-tory syncytial virus (RSV) (reviewed in [3]). In older children [2] and adults [4, 5], human rhinoviruses (HRVs) are implicated in the majority of cases, with variable contributions from human coronaviruses (HCoVs), influenza viruses, parainfluenza viruses, RSV, and human metapneumovirus (HMPV) (reviewed in [6]). However, the factors that determine the clinical outcomes associated with RTIs in persons with asthma are not well understood. Host factors governing in-flammatory and immune responses have been dem-onstrated to influence whether a host with asthma ex-periencing a viral RTI will develop an exacerbation of symptoms [7–10]. In contrast, it remains unresolved whether variation in viral pathogens may also influence this outcome. For example, despite the prominent as-sociation between HRVs and asthma exacerbation, de-liberate inoculation of a laboratory-adapted HRV-16

isolate into subjects with asthma did not produce wheezing, despite efficient infection and the generation of upper respiratory tract symptoms [11–16]. Although these experimental HRV inoculation studies have not demonstrated clinically significant exacerbations, a small decline in pulmonary function has been observed [17, 18]. Likewise, not every cold in subjects with asthma is associated with exacerbation of asthma symptoms. These findings raise the possibility that some respiratory viral pathogens may have greater potential than others of triggering asthma exacerbations in susceptible hosts.

If this is so, then the spectrum of viral isolates recovered from RTIs in persons with asthma who experience exacerbations should differ from that in persons with asthma whose RTIs do not trigger exacerbations. Few prior studies have attempted to make this comparison, and those that did have generally not used methods that can differentiate among the different subtypes of viruses within a family. Estimating the number of HRV isolates, rather than identifying them by serotype or genotype, may be insufficient given that certain isolates of HRV are demonstrably inefficient at triggering asthma on inoculation.

Part of the reason for the paucity of prior studies of this type derives from the limitations of conventional viral detection methods. Viral culture is insensitive, and antigen testing, while reasonably sensitive, is (1) unavailable for several important classes of pathogen, notably HRVs and HCoVs, and (2) not designed to discriminate among isolates of a given viral species. Polymerase chain reaction (PCR) methods have high sensitivity but are limited to the detection of previously characterized or highly conserved viral sequences.

We have developed an alternative, comprehensive strategy for viral detection that uses the Virochip, a DNA microarray bearing the most conserved sequences of all known viruses of humans, animals, plants, and microbes [19, 20]. In addition to the ability of the Virochip to detect known viruses, it can also detect new members of known virus families by cross-hybridization—a significant advantage over PCR-based methods [20–23]. Here, we have applied the Virochip to an ongoing prospective study designed to analyze the diversity of viruses that cause RTIs in adults with and without asthma, with particular attention given to those that provoke asthma attacks.

SUBJECTS, MATERIALS, AND METHODS

Subject recruitment. We recruited adults with and without asthma by advertising on a community Web site (craigslist; http://sfbay.craigslist.org/), by posting flyers on campus, and by sending e-mail messages to subjects who had responded to previous advertisements for research studies. Participant recruitment started in the fall of 2001 and extended through the end of December 2004. The announcements requested that subjects contact us within 48 h of the onset of "cold" symptoms. We evaluated subjects first within 1–3 days of cold onset (visit 1), then again between days 4 and 7 of cold symptoms (visit 2), and at 6 weeks or longer thereafter to assess baseline status (visit 3). Eighty-three subjects were enrolled; 53 had asthma, and 30 did not. Asthma was defined as a history of asthma symptoms (recurrent dyspnea, wheezing, and chest tightness) associated with a positive methacholine test result [8]. Subjects without asthma had neither a history of asthma symptoms nor a positive methacholine test result. All were informed of the purposes, procedures, and risks of participating, and all signed informed-consent forms approved by the University of California, San Francisco, Committee of Human Research.

Study procedures. At the first visit, participants completed questionnaires on demographics, medical history, asthma history, and date of onset of cold symptoms. At each visit, participants provided information on the severity of current upper and lower respiratory tract symptoms by use of a validated cold questionnaire [24] and diary form [8] that have been described elsewhere. Spirometry measurements (forced expired volume in 1 s [FEV_1] and forced vital capacity [FVC]) were obtained, and the percentage predicted was calculated using Crapo equations. Nasal lavage (NL) was then performed by instilling 5 mL of warmed normal saline into each nostril and, after a 20-s dwell time, having the subject expel the nasal contents into a plastic cup. Subjects with asthma recorded symptom severity and A.M. and P.M. peak flow measurements (AirWatch; iMetrikus) in a diary twice daily for 7 days after visit 1 and 7 days before visit 3. The visit procedures were repeated at day 5–7 and at baseline (6 weeks or longer) after the onset of cold symptoms.

Definition of asthma exacerbation. Before initiating the study, we defined asthma exacerbation as either a worsening of asthma symptoms that required corticosteroids (systemic or topical) or a worsening of symptoms on the basis of study measurements. The latter was defined by an increase in daily asthma symptom score of 10 points or more (range, 0–50 points) above the baseline daily average for at least 2 days together with at least 1 of the following: (1) decrease in FEV_1 by 10% or more at any of the first 2 visits; (2) decrease in peak flow measurements by 20% or more for at least 2 days during the cold week; or (3) increase in daily albuterol use of 4 puffs or more for at least 2 days during the cold week.

Specimen processing. NL specimens from visit 1 (days 1–3 after the onset of cold symptoms) were analyzed for the presence of virus by PCR, viral culture, and Virochip microarray analysis. For microarray analysis and PCR, 1 mL of homogenized NL specimen was mixed with 3.5 mL of RLT buffer (Qiagen) containing 2-mercaptoethanol and then frozen at bedside in dry ice and stored at −80°C.
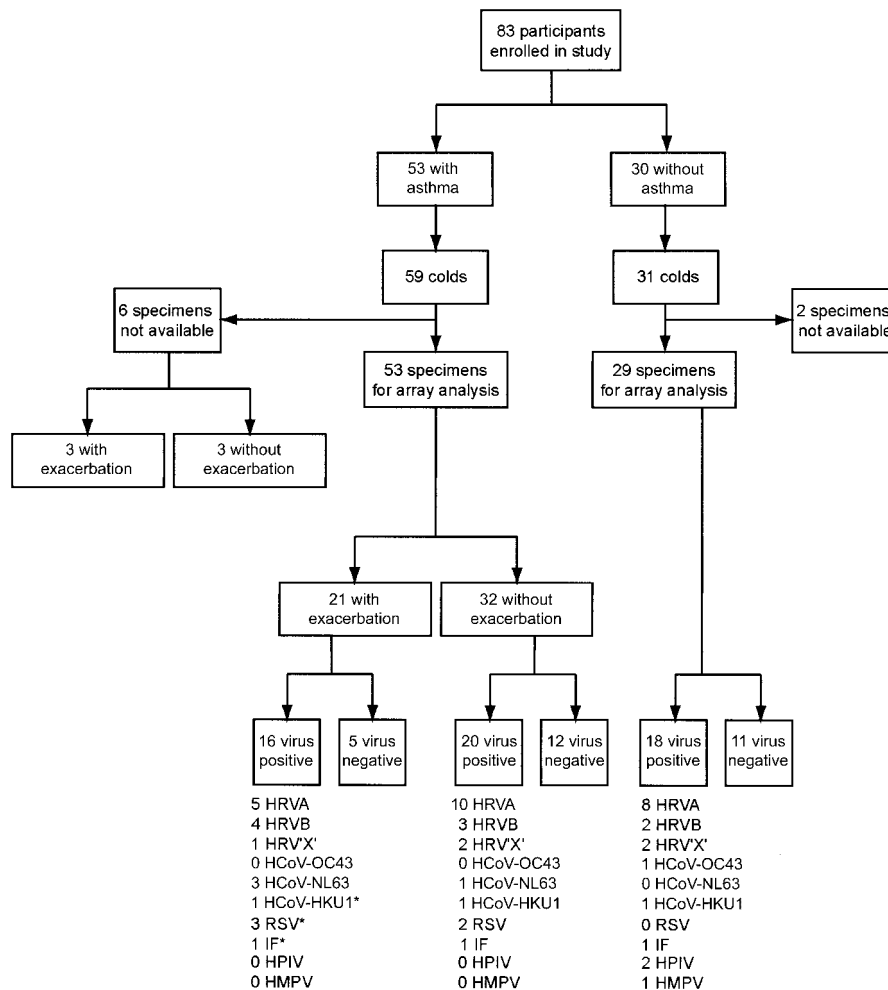
Figure 1. Summary of study participant distribution and Virochip results. Asterisks indicate viruses detected in specimens containing double infections. HRV'X' is a divergent human rhinovirus (HRV) subgroup identified by array and sequence analysis. HCoV, human coronavirus; HMPV, human meta-pneumovirus; HPIV, human parainfluenza virus; IF, influenza virus; RSV, respiratory syncytial virus.

Viral culture isolation. A 0.1-mL NL aliquot was cultured in duplicate with a combination of 5 different cell lines (HeLa, WI38, MRC5, primary monkey kidney, and HFDL, an in-house line of human fetal diploid lung cells [California Department of Health Services Viral and Rickettsial Disease Laboratory, Richmond]). Some specimens were inoculated into all 5 cell types, and all were cultured in at least 3, including primary monkey kidney and human fetal diploid. If cytopathic effect occurred, viral antigen detection tests (Respiratory Viral Antigen Detection Kit; Chemicon) were performed to identify antigens from adenovirus, RSV, influenza viruses A and B, and parainfluenza viruses 1, 2, and 3. Enteroviruses and rhinoviruses were differentiated by reactivity with the monoclonal pan-enterovirus reagent from Chemicon and the enterovirus monoclonal antibody from Dako Cytomation (enterovirus clone 5-D8/1).

RNA extraction. Samples were masked and total RNA was extracted with an on-column recombinant DNase treatment step using the RNeasy Miniprep Kit (Qiagen), in accordance with the manufacturer's instructions.

Amplification and microarray analysis. RNA was randomly amplified and labeled and was then hybridized to the Virochip microarray as described elsewhere (protocol S1 in Wang et al. [19]). Microarrays were scanned using the Axon 4000B scanner and GenePix software (version 3; Axon Instruments). A.K., who was blinded to the viral culture isolation and HRV PCR results, interpreted the hybridization signatures. Epredict, a computational tool developed for the Virochip array hybridization signature, was used to determine the Virochip results, with the P value cutoff for positivity set at .05, as described elsewhere [25]. Samples for which multiple viruses met this threshold were further evaluated by hierarchical cluster analysis of sum-normalized background-subtracted array hybridization intensities from all NL specimens, by use of Cluster
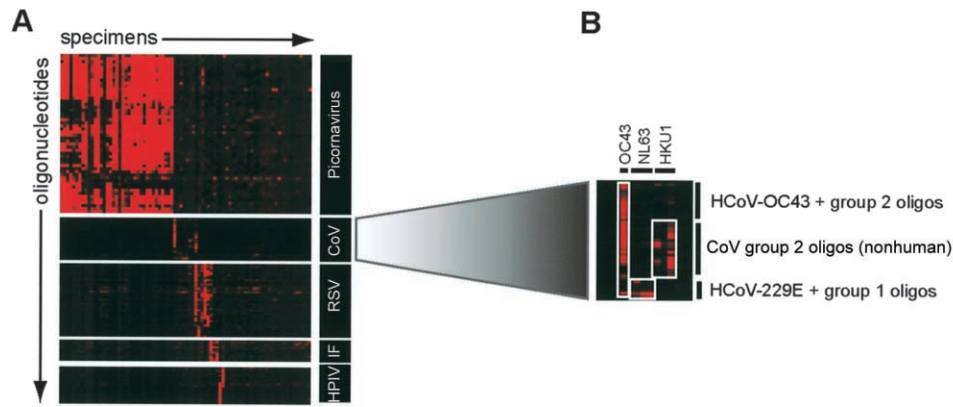
Figure 2. Clustogram of Virochip hybridization signatures. *A,* Nasal lavage specimens (*X*-axis) clustered according to the sum-normalized fluorescent intensity of array signal, with major viral oligonucleotide clusters (*Y*-axis) identified at right. *B,* Zoomed-in view of the 3 human coronavirus (HCoV) signatures detected by the Virochip. Each hybridization signature is boxed in white, with its corresponding HCoV type (OC43, NL63, and HKU1) indicated at the top of the clustogram; the identity of oligos lighting up within the clusters are indicated at right. HPIV, human parainfluenza virus; IF, influenza virus; RSV, respiratory syncytial virus.

software (version 3) [26]. Co-occurrence of samples within clusters was used to make calls for specimens that either had multiple significant Epredict scores or had no significant Epredict scores. All viral-positive calls were confirmed by recovery of viral sequence.

Specific PCR for HRV detection. For each sample, 3 μL of the randomly amplified material was used for independent PCR to detect and sequence the HRV VP4/VP2 capsid gene junction, as described elsewhere [27]. The VP4/VP2 PCRs were performed in a blinded manner. Primers 9656-reverse (5′-GCATCIGGYAR-YTTCCACCACCANCC-3′; positions 1083–1058 of HRV-1b; National Center for Biotechnology Information [NCBI] accession number D00239) and 9895-forward (5′-GGGACCAACTACTT-TGGGTGTCCGTGT-3′; positions 534–560 of HRV-1b) were used for PCR (35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s).

Comparative sequence analysis of recovered HRV VP4/VP2 PCR products. ClustalW (version 1.82) was used to align the VP4/VP2 capsid gene junction sequences obtained for the clinical isolates of HRV for all 102 reference HRV serotypes [27]. Neighbor-joining phylogenetic trees were generated from the resulting alignment using the PHYLIP package (version 3.2) [28].

Specific PCR for HCoVs. For each of the 8 HCoV-positive samples identified by Virochip microarray analysis, 3 μL of the randomly amplified material was used for PCR to detect a 440-bp region of the polymerase gene using pan-CoV primers (5′-GGTTGGGACTATCCTAAGTGTGA-3′ and 5′-CCATCATCA-GATAGAATCATCATA-3′) that have been described elsewhere [29]; amplification was with 40 cycles of 94°C for 60 s, 48°C for 60 s, and 72°C for 60 s.

Sequence analysis of HCoV PCR products amplified from clinical isolates. PCR products were extracted using QIA-quick (Qiagen) and were either sequenced directly using path-

ogen-specific primers or subcloned into pCR2.1 TOPO vector (Invitrogen) and sequenced using M13 forward and M13 reverse primers with the BigDye Cycle Sequence Kit on an ABI 3130 automated sequencer (Applied Biosystems). The identity of each of the HCoVs in the present study was inferred on the basis of the highest scoring match from BLAST analysis (version 2.2.13) [30] of the resulting sequences.

Genome sequence recovery from HRV'X'-1 and HRV'X'-2. Amplified cDNA was subcloned into pCR2.1 TOPO plasmid (Invitrogen). Three hundred eighty-four colonies were picked, and DNA was purified by magnetic bead isolation followed by DNA sequencing using the BigDye Cycle Sequence Kit/ABI 3730xl sequencer. Sequence reads were assembled by use of CONSED for Linux (version 13.4) [31]. Assemblies were screened by BLAST analysis [30] to remove any contigs with human or bacterial sequence similarity. Gaps in assemblies were filled by synthesis of oligonucleotides with at least 100 bp of overlapping sequence with available contigs.

Accession numbers. GenBank accession numbers for the sequenced viruses presented here are EF077237-EF077281. The GEO database (http://www.ncbi.nlm.nih.gov/geo/) series accession number for all Virochip microarray data presented here is GSE8053.

RESULTS

Performance of the Virochip relative to conventional viral detection methods. The first goal of our analysis was to assess the performance of the Virochip relative to conventional viral detection methods. To do this, we used a set of NL specimens from an ongoing prospective study of naturally acquired upper RTIs (NatURIs) in adults with and without asthma. A total of 82 cold events captured from this study were available for anal-

Table 1. Detection rates for and viruses detected by culture and the Virochip microarray.

Viral detection	Culture	Virochip
Samples viral positive, total no.	14	53
Samples viral negative, total no.	68	28
Samples tested, total no.	82	82
Overall detection rate, %	17	65
Human rhinovirus	14	37
Human coronavirus	0	8[a]
Respiratory syncytial virus	1[b]	5[b,c]
Influenza virus	2[a]	3[a]
Human parainfluenza virus	0	2
Human metapneumovirus	0	1
Multiple viruses	1	2

[a] Respiratory syncytial virus also detected in 1 specimen.
[b] Influenza virus also detected in 1 specimen.
[c] Human coronavirus also detected in 1 specimen.

ysis. Breakdown of participants, specimens, associated clinical outcomes, and corresponding Virochip results are summarized in figure 1. Each NatURI specimen was analyzed independently in a blinded manner by 3 distinct viral detection methods: Virochip analysis and culture isolation for 9 common respiratory pathogens (HRV; RSV; influenza viruses A and B; human parainfluenza viruses 1, 2, and 3; adenovirus; and human enterovirus) and PCR for HRV. Where the Virochip detected viruses, follow-up PCR and sequence recovery was performed to confirm the presence of the detected viral species.

A high proportion of specimens tested positive for virus by Virochip analysis (65%). Reflective of the outpatient setting of our study, the 2 most prevalent virus families detected by the Virochip corresponded to HRVs and HCoVs (figure 2A and table 1). Four additional viruses generally thought to be associated with lower RTIs—RSV and the closely related HMPV, influenza virus, and human parainfluenza virus—were also detected. We also detected double infections (RSV plus HCoV and RSV plus influenza virus) (figure 2A and table 1).

In contrast, virus was detected by culture isolation in only 17% of samples. Three different viral pathogens (HRV, RSV, and influenza virus) and 1 double infection were detected by this method (table 1). Head-to-head comparison of these 2 assays yielded an overall low concordance (55%). Virtually all of the discordant results (34/35) corresponded to results that were positive by the Virochip but negative by culture isolation.

These results were not surprising, given the known limitations of viral culture isolation [32]. We also compared the performance of the Virochip to pathogen-specific PCR, which is known for its high sensitivity. To address this in a statistically significant manner, we focused on PCR detection of HRV [27], the most common pathogen detected in the study population. We found excellent concordance (98% agreement) between Virochip and HRV-specific PCR results, indicating that Virochip

analysis is a highly sensitive (97%) and specific (98%) viral detection method in comparison to pathogen-specific PCR (table 2).

Detection of both known and novel HCoVs by the Virochip. Analysis of Virochip hybridization signatures for the 8 HCoVs detected among the study subjects revealed 3 distinct signatures (figure 2B). PCR recovery and sequence analysis of a fragment of the HCoV polymerase gene [29] revealed that each of these signatures corresponded to a distinct HCoV subtype: (1) HCoV-OC43, (2) HCoV-NL63, and (3) HCoV-HKU1. Each of these isolates shared between 97% and 100% sequence identity with the corresponding HCoV polymerase gene sequences present in the NCBI database.

Surprisingly, the bulk of the HCoVs detected in this outpatient population did not correspond to the more common HCoV types (OC43 and 229E) historically detected in US adult RTIs [33]. Of the 8 HCoVs detected in this study population, 4 were HCoV-NL63, 3 were HCoV-HKU1, and only 1 was HCoV-OC43. The different HCoV types were detected in samples collected at different times during the study. All 4 of the HCoV-NL63 isolates were detected in persons with colds that occurred during the early winter of 2002, whereas the HCoV-HKU1 isolates were detected during the late fall of 2001 (1 isolate) and the early winter of 2004 (2 isolates). The sole HCoV-OC43 isolate was identified during the mid-fall of 2004. No HCoVs infections were detected in this study population in 2003. Interestingly, HCoV-NL63 was detected only in persons with asthma, and 3 of the 4 infections were accompanied by an exacerbation of asthma symptoms.

Detection of a divergent subgroup of HRVs by the Virochip. The most commonly detected virus in our study population was HRV (figures 1 and 2A and table 1). To investigate HRV diversity, we sequenced the junction of the VP4/VP2 capsid genes of the HRV isolates and performed phylogenetic analysis of these sequences and of the published sequences from all 102 HRV serotype reference strains [27]. We found that the Virochip hybridization signatures we observed corresponded to ~29 different HRVs: 16 HRVA serotypes, 8 HRVB serotypes, and a novel third set of 5 divergent HRVs (referred to as HRV'X'; figure 3), which possessed slightly more sequence similarity to HRVA than to HRVB reference serotypes.

None of the divergent HRV'X' isolates were culturable; thus,

Table 2. Agreement between polymerase chain reaction (PCR) and the Virochip microarray for detection of rhinovirus.

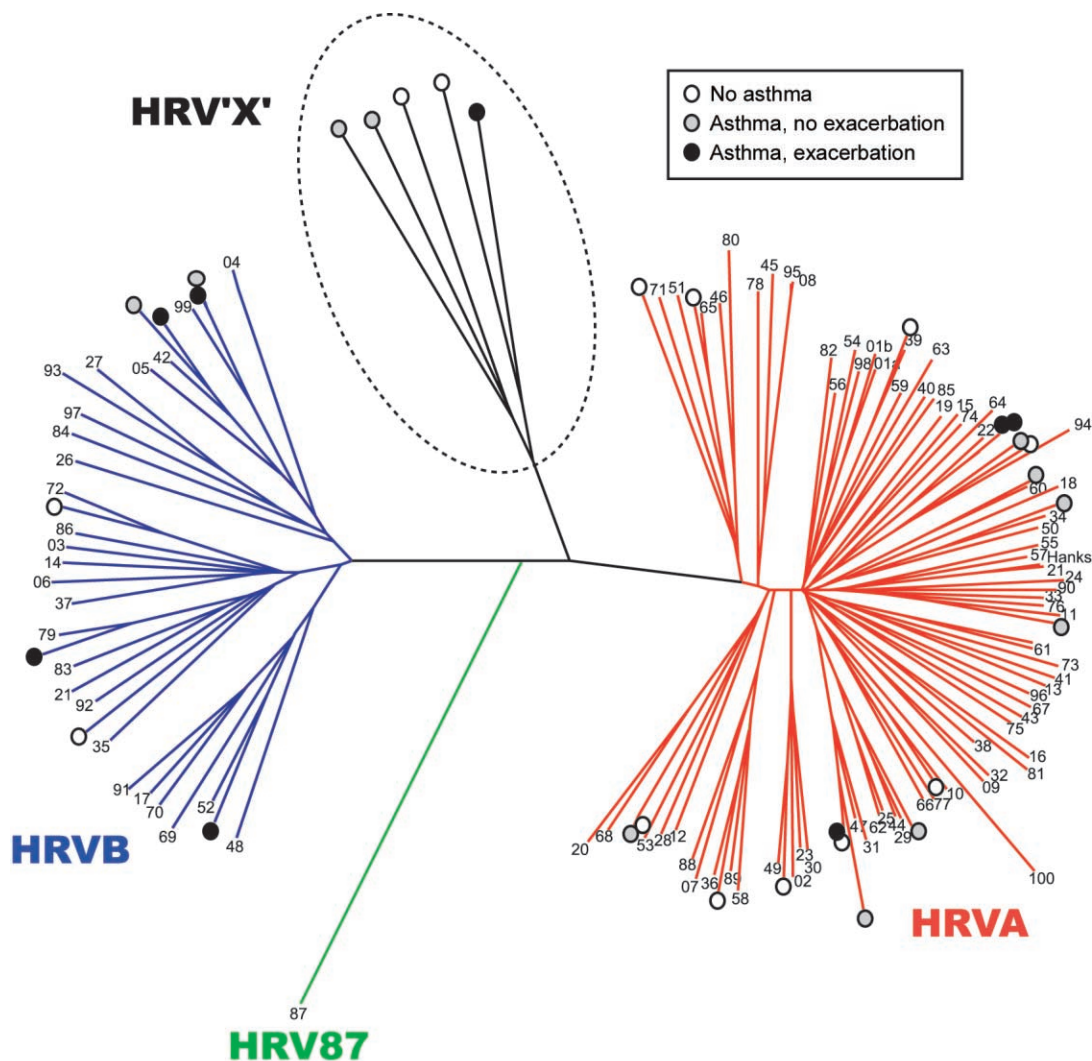| Result	PCR positive	PCR negative	Total
Virochip positive	36	1	37
Virochip negative	1	43	44
Total	37	44	81

Figure 3. Phylogenetic grouping of human rhinovirus (HRV) VP4/VP2 sequences. Red indicates HRVA subgroup members, blue indicates HRVB subgroup members, green indicates an HRV87 rhino/entero outlier, and the dashed circle highlights a branch of divergent HRV isolates (HRV'X'). The nos. at the ends of the branches indicate HRV reference serotype identifiers, and the circles at the ends of branches indicate the participant type and clinical outcome accompanying the cold event for clinical isolates.

unambiguous detection and classification of these isolates by conventional serotyping [34], drug susceptibility [35], or receptor-type usage assays [36] was not possible. Recovery of complete coding sequence from 2 of the HRV'X' isolates (HRV'X'-1 and HRV'X'-2) and analysis of their sequence identity with a representative subset of 27 fully sequenced HRVA subgroup genomes and 7 fully sequenced HRVB subgroup genomes [37] indicated that these HRV'X' isolates were indeed HRVs.

Scanning pairwise identity revealed that the differences between the HRV'X' and the HRVA subgroup genomes were not confined to a single locus but spanned the entire genome (figure 4). Although the VP4/VP2 phylogenetic analysis indicated that the HRV'X' isolates were more similar to HRVA than HRVB

reference serotype strains, comparison of the level of genome-wide sequence identity shared within the fully sequenced subset of HRVA genomes to the levels of sequence identity shared between the HRVA and the HRV'X' isolates showed that the HRV'X' isolates were almost as genetically distinct from HRVA as the HRVB subgroup genomes (figure 4). Moreover, pairwise sequence identity between the HRV'X' genomes was much lower than that detected among the fully sequenced HRVA or HRVB subgroup genomes (data not shown). Taken together, these data demonstrate that these HRV'X' isolates correspond to a novel divergent subgroup of HRV and suggest that this divergent branch of HRV'X' isolates may possess a higher level of genetic diversity than seen previously in the HRVA and HRVB subgroups.
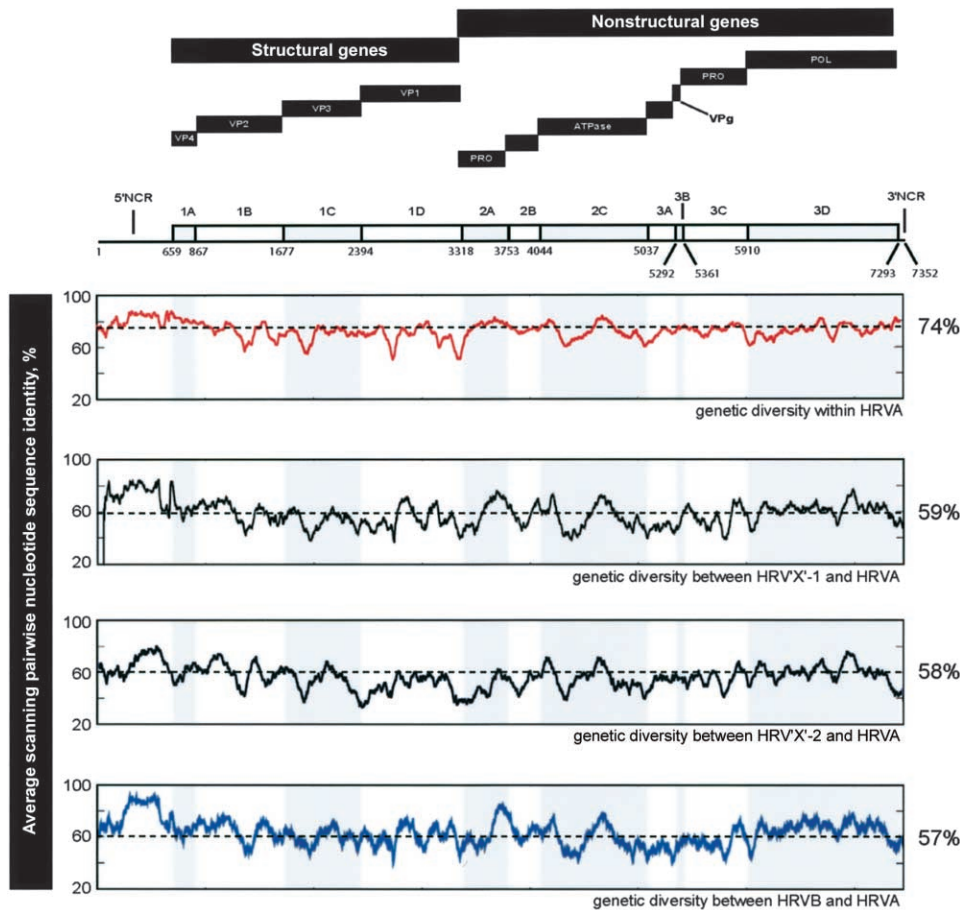
Figure 4. Comparison of genomewide pairwise nucleotide sequence identity of human rhinovirus (HRV) A, HRVB, and HRV'X' genomes. *Top,* HRV genome organization. Black bars above the genome schematic indicate classes of gene products and gene product identities, where known (ATPase, DEXH-box ATPase protein; NCR, noncoding region; POL, RNA-dependent RNA polymerase; PRO, viral protease; VP, viral protein; VPg, viral protein genomic encoding the 5′ protein that caps the viral genome). Gray shading of every other gene in the genome is provided for orientation in the lower panels. Coordinates for gene boundaries derived from alignment of 34 HRV reference genomes are shown below the genome schematic. The average percentage pairwise nucleotide identity scans were performed using a window of 100 nt, advanced in single-nucleotide steps across the genome. The red plot shows a representative subset of HRVA genome sequences ($n = 27$), the black plots show fully sequenced HRV'X' genomes compared with HRVA genomes, and the blue plot shows fully sequenced HRVB ($n = 7$) and HRVA ($n = 27$) genomes. Dashed lines and the percentages shown at right indicate the overall genomewide average pairwise nucleotide identity for each comparison shown.

DISCUSSION

This is the first prospective study to use a pan-viral detection strategy to investigate the influence of viral pathogens on clinical outcome in RTIs in persons with asthma. We find that, like PCR, the Virochip technology is superior to standard culture isolation methods for detection of viral pathogens. Moreover, the Virochip exhibits comparable sensitivity and specificity to pathogen-specific PCR. On the whole, the distribution and proportion of distinct viral pathogens detected by the Virochip agrees with previous PCR-based analyses of viral pathogens associated with upper RTIs and those accompanied by exacerbation of asthma symptoms [3, 38]. However, Virochip analysis has allowed us to uncover a remarkable amount of

diversity among the viral pathogens in this relatively small study population. This diversity indicates that future studies that seek to link a particular virus or set of viruses to a discrete clinical outcome, such as exacerbation of asthma symptoms, will need to include large numbers of subjects and use pan-viral detection methods (such as the Virochip) that can differentiate among such isolates.

Two observations of viral diversity uncovered by Virochip analysis of NL specimens derived from this study are particularly noteworthy. First, the diversity and distribution of HCoVs detected in the present study were surprising. The 2 more recently described HCoV-HKU1 and HCoV-NL63 were the predominant HCoVs rather than HCoV-OC43 and HCoV-

229E, which have been traditionally implicated in up to 15% of common colds in the US adult population [32]. Here, instead we see HCoV-NL63 and HCoV-HKU1 making up approximately that same proportion of colds detected in our study population. Given that HCoV-NL63 and HCoV-HKU1 have not been implicated previously as significant players in outpatient respiratory tract illnesses among immunocompetent adults in the United States [39, 40], these results were unexpected. No HCoV-229E isolates and only a single HCoV-OC43 isolate were detected in this study group, despite the fact that independent studies with the Virochip have demonstrated that, when present, both HCoV-OC43 and HCoV-229E are readily detectable (C. Y. Chiu, A. Urisman, T. L. Greenhow, submitted). Further analysis will be required to determine whether the patterns of HCoVs detected here reflect an increased susceptibility of adults with asthma to contract these HCoVs, the arrival of a local outbreak of these HCoV types, or an actual shift in the prevalence of the distinct HCoVs circulating in the US adult population.

Second, the Virochip detected remarkable and unanticipated diversity among HRV isolates. In addition to detecting almost 30 distinct HRV species closely related to known reference HRV serotypes, we also identified a subgroup of genetically distinct HRVs in a significant fraction (5/37) of the clinical isolates of HRV. Although the clinical significance of HRV diversity remains incompletely understood, the detection of such a high level of genetic divergence among HRV strains captured in this relatively small study population indicates that the standard HRV reference serotypes (which were characterized almost 30 years ago) do not adequately describe the diversity of currently circulating HRVs.

We do not believe that these HRV'X' strains are an anomalous subgroup of HRVs unique to this study population because (1) a similar proportion of divergent HRV strains were detected by Virochip analysis in an unrelated cohort of pediatric subjects with RTIs (C. Y. Chiu, A. Urisman, T. L. Greenhow, submitted); (2) divergent HRVs have also been recently identified in a study of pediatric respiratory infections in Australia [41]; (3) based on the 5′ noncoding region sequence alone, virtually identical isolates of HRV have been reported in independent analyses of HRV infections among Europeans [42]; and (4) recent independent application of a distinct pan-viral detection tool, MassTag PCR analysis, has also documented a set of highly diverged HRVs circulating in the US population [43].

Comparison of the VP4 sequences of the HRV'X' strains identified here suggests that one of the HRV'X' strains (HRV'X'-2) possesses high sequence similarity (98% identity with VP4 sequence DQ875926) to one of the divergent HRVs recently reported by Lamson et al. [43]. However, the VP4 sequences in the other 4 HRV'X' strains identified here possess <85% nucleotide sequence identity with the set of divergent HRVs

identified by Lamson et al. [43]. The identification of 2 distinct sets of genetically divergent HRV clinical isolates indicates that the set of previously unrecognized HRVs currently circulating in the United States may be quite large. A deeper knowledge of the extent of the current HRV diversity should inform future studies of the role played by HRV strains in asthma exacerbations, given that a high proportion of such events are attributable to infection by these agents.

In sum, the present data demonstrate that the Virochip captures the entire spectrum of known respiratory viral pathogens in a single test, exhibits excellent sensitivity, and provides the capacity to identify as-yet-undiscovered agents. The application of the Virochip to prospective clinical studies should enable us to develop a comprehensive picture of the diversity of viral pathogens present during infection, a critical missing piece of the puzzle required to advance our understanding of how different viral pathogens influence the course and spectrum of disease.

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